Temporal Analysis of Cotton Boll Symptoms Resulting From Southern Green Stink Bug Feeding and Transmission of a Bacterial Pathogen

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ABSTRACT The southern green stink bug, *Nezara viridula* (L.), is a significant pest of cotton, *Gossypium hirsutum* L., and is becoming an increasing challenge due to the decrease in use of broad-spectrum insecticides on the crop. The southern green stink bug can vector an opportunistic *Pantoea agglomerans* strain (designated Sc 1-R) into cotton bolls, resulting in infection. The appearance of stink bug damage varies, and pest managers cannot readily identify its source. This research reports a systematic depiction of green, immature boll responses at various stages of maturity (1, 2, and 3 wk post-anthesis [WPA]) to stink bug injury and to infection by the vectored cotton pathogen by demonstrating the progression of effects 1, 2, and 3 wk after exposure (WAE). When laboratory-reared adult southern green stink bug not harboring Sc 1-R deposited bacteria into greenhouse-grown bolls at 1, 2, or 3 WPA during feeding/probing, bacteria reached concentrations of 10^9, 10^9, and 10^3 colony-forming units (CFUs)/g tissue, respectively, at 3 WAE, yet caused minimal seed and lint damage regardless of the age of the bolls that were penetrated. Bolls at a maturity of 1 or 2 WPA showed similar susceptibility when exposed to stink bugs that vectored Sc 1-R. After a week of infection, seeds were salmon-pink with normal white lint and up to 10^4 CFUs/g tissue when Sc 1-R was detected. Necrosis of the entire inoculated locule(s) with a maximum Sc 1-R concentration detected at 10^8 CFUs/g tissue occurred in samples harvested 2 or 3 WAE. Conversely, seed and lint deterioration due to the transmitted opportunist into bolls exposed 3 WPA was confined to the puncture site. In summary, after a week of development, bolls were tolerant to southern green stink bug feeding/probing damage and to nonpathogenic bacteria, but they were severely damaged when the opportunistic pathogen Sc 1-R was transmitted. At 3 WPA, the fruit was immune to the spread of the pathogen with infections confined to the puncture site.

KEY WORDS stink bugs, vector, cotton bolls, seed rot, bacterial opportunist

Damage to developing cotton, *Gossypium hirsutum* L., bolls by insects that feed via a piercing/sucking mechanism has increased over the past several years due to changes in pest management (Barbour et al. 1990; Bundy and McPherson 2000). Successes of the national boll weevil, *Anthonomus grandis grandis* Boheman, eradication program, and planting of transgenic cotton cultivars expressing the *Bacillus thuringiensis* toxin to control the heliothine complex [bollworm, *Helicoverpa zea* (Boddie)/tobacco budworm, *Heliothis virescens* (F.)] has resulted in a decreased number of applications of broad-spectrum insecticides (Greene et al. 1999; Bundy and McPherson 2000; Jackson et al. 2004). Consequently, infestations by formerly minor pests have risen (Panizzi et al. 2000; Greene et al. 2001; Willrich et al. 2004a). For both 2005 and 2006, Williams (2007) reported that the heliothine complex was the most significant cotton pest, with lygus bugs (Heteroptera: Miridae) second, and stink bugs (Heteroptera: Pentatomidae) third.

Seed and boll rots caused by nontraditional pathogens are another emerging phenomenon that has significantly decreased yields of cotton grown particularly in southeastern cotton belt states (Hudson 2000, Hollis 2001). In 1999, South Carolina cotton fields were afflicted with a previously unobserved disease that caused yield losses from 10 to 15% (Hudson 2000). Diseased green bolls from affected fields externally seemed normal; yet, transverse cross-sections revealed one or more locules exhibiting necrotic inner carpel, seed, or lint tissue (Edmisten 1999). Infected locules produced dark, matted lint with dead, flattened seeds instead of normal white fluffy fiber and mature seeds.

An example of a recently documented cotton pathogen is an opportunistic *Pantoea agglomerans* strain that is an agent of boll rot based on fulfillment of Koch's postulates (Medrano and Bell 2007) using several infective isolates recovered from diseased bolls from a South Carolina field collection. When introduced through a puncture that breached the carpel wall, a laboratory-selected, rifampicin (Rif)-resistant variant

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(strain Sc 1-R) of a *P. agglomerans* field isolate caused disease symptoms that resembled those of infected bolls collected from the field. Infections required an introduction of the opportunist Sc 1-R through the endocarp, and they did not result from inoculations on the surface of the fruiting body or by introduction of the mutant into the petiole vasculature. We hypothesized that field infections involved an insect vector that employs a piercing/sucking mechanism to feed on bolls.

Recently, we reported that southern green stink bug, *Nezara viridula* (L.), feeding/probing readily results in the transmission of bacteria into cotton bolls (Medrano et al. 2007). Bolls were tolerant to both stink bug feeding and insect-transmitted nonpathogenic microorganisms that caused negligible damage to the seed and lint. Conversely, vectoring of the pathogenic Sc 1-R strain resulted in complete locule destruction with symptoms that consisted of discolored fiber and necrotic seed. Our established disease model, using the southern green stink bug as a vector, permitted the systematic analysis of the infection's progression and symptom expression. Here, we discuss the vulnerability of bolls to stink bug infestation and a southern green stink bug-vectored pathogen (*P. agglomerans*) at several stages of boll maturity when exposed, and the presence/absence of disease symptoms at three weekly intervals after exposure to insects on bolls of determined age.

Materials and Methods

Exposure of Insects to *P. agglomerans*. Feral adult southern green stink bugs collected from a commercial soybean, *Glycine max* (L.) Merr., field in College Station, TX, were used to establish a laboratory colony. Reared insects at all life stages were maintained in an incubator at 28°C and a photoperiod of 14:10 (L:D) h. Colony adults and nymphs were fed fresh green beans, *Phaseolus vulgaris* L., that were replenished every 2–3 d. The green beans were thoroughly washed in a 5% $NaHCO_3$ solution before being provided to the insects. A mutant *P. agglomerans* strain (Sc 1-R) determined previously to be vectored by stink bugs, was used as the cotton pathogen (Medrano and Bell 2007). Strain Sc 1-R was maintained on Luria Bertani agar (LBA; Difco, Detroit, MI) amended with Rif (100 μg/ml; LBA Rif) and incubated at 28°C.

Eighteen-hour cultures of Sc 1-R were used to prepare suspensions in sterile water and adjusted spectrophotometrically (A_{600} = 1.0). The bacterial inoculum consisted of a 1 ml Sc 1-R suspension that was diluted in 49 ml of sterile water. Green beans used for the vectoring experiments were first sterilized (121°C; 20 min; 1 kg cm^2) and then cross-sectioned into ≈3-cm portions using a sterile scalpel (bean ends were discarded). Quality control methods to ensure bean sterility are described in Medrano et al. (2007). Bean sections were soaked for 2 min in either the Sc 1-R inoculum or sterile water and then blotted dry with sterile paper towels. A treated bean portion was aseptically transferred into a sterile disposable petri dish (100 by 15 mm) along with a sexed stink bug. After 2 d of incubation with the treated bean, all bugs were transferred into fresh dishes containing a sterile green bean section. After two more days, the replenishment procedure was repeated and the insects were incubated for three more days before caging with the bolls.

Insect Infestation on Cotton Bolls. Cotton plants ('Acala Maxxa') were grown in the greenhouse from seed by using the methods and parameters reported in Medrano and Bell (2007). Insect control measures are described in Medrano et al. (2007). Representative boll samples were tested for the presence of Rif-resistant bacteria on the carpel by imprinting on LBA amended with Rif. Bacterial growth was determined after a 2-d incubation period at 28°C. Bolls of the appropriate age (i.e., 1, 2, or 3 wk post anthesis [WPA]) were individually enclosed in cages as described in Medrano et al. (2007). A single control (i.e., not exposed to Sc1-R; untreated) or treated stink bug (i.e., exposed to Sc1-R; treated) was placed in each cage and held for 2 d. A supplementary negative control consisted of bolls caged without an insect. After the caging period, live insects were harvested and internal bacterial concentrations were determined as described below.

For surface sterilization, insects were independently placed in a 14 ml round bottom Falcon tube (BD Biosciences, Franklin Lakes, NJ) that contained 70% ethanol (10 ml) and were gently inverted for 8 min. Next, the insects were rinsed twice for 1.5 min with 25 ml of sterile water followed by separately placing them into a 1.1-ml microtube (SPEX SamplePrep, Metuchen, NJ) that contained 0.5 ml of PO_4 buffer (0.1 mol/liter, pH 7.1) and a sterile 4-mm stainless steel ball (SPEX SamplePrep). After addition of the insect, an identical steel ball was added to the tube that was then capped. The 1.1-ml microtubes were arranged in strips of eight and then placed in a 96-tube rack for pulverization. The wash water was tested for sterility by using the methods discussed in Medrano et al. (2007). The insects were ground for 5 min at 1500 strokes/min by using a 2000 Geno/Grinder (SPEX SamplePrep), and then 10-fold dilutions (PO_4 buffer, pH 7.1) were plated on both LBA and LBA amended with Rif. After 2-d incubation at 28°C, bacterial colonies were counted and expressed as colony-forming units (CFUs) per insect.

Cotton Boll Evaluation. Insect cages were resealed after removal of the stink bugs and the known-age cotton bolls were harvested 1, 2, or 3 wk after exposure (WAE) to the insect or grown to yield. Green bolls were individually surface sterilized for 10 min in a 0.5% sodium hypochlorite solution and then rinsed for 2 min in 25 ml of sterile water three times. The carpel wall was excised with a sterile scalpel. Evidence of insect feeding was recorded based on inner carpel blistering, as well as lint and seed tissue damage. Lint and seed (≈0.5 g) from locules with signs of insect feeding were diced, transferred into a 1.1-ml microtube, and pulverized for 10 min as described above. Seed and lint tissue from bolls caged without a stink bug were processed as negative controls. Bacterial colonies were counted and CFUs per g locule tissue were calculated after 2 d of incubation at 28°C.

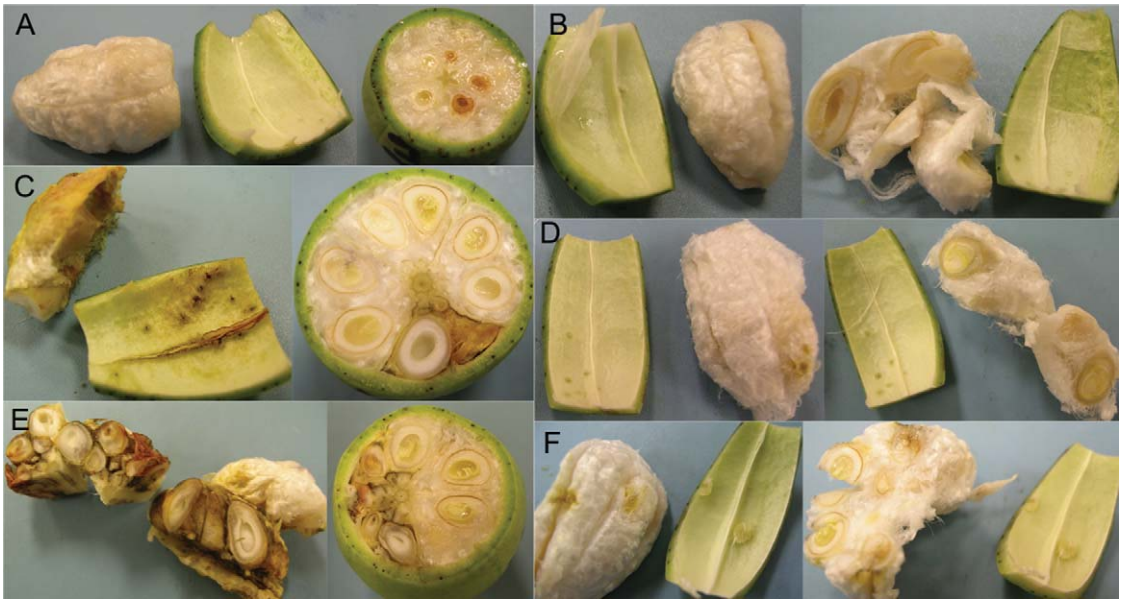

Fig. 1. Temporal effects of feeding/probing on greenhouse-grown cotton bolls by *N. viridula* infested with strain Sc 1-R or not harboring the bacterial cotton pathogen. Bolls were individually caged with a stink bug for 2 d at 1 wk post-anthesis. Samples were photographed at 1 (A and B), 2 (C and D), or 3 (E and F) wk after exposure to an insect. A, C, and E illustrate infection progression resulting from Sc 1-R transmission by stink bugs initially exposed to the opportunist via a contaminated food source. B, D, and F illustrate damage caused by stink bugs not harboring a cotton pathogen.

Results

Exposure of Insects to *P. agglomerans*. A preliminary analysis using 16 stink bugs (eight Sc 1-R contaminated and eight control insects) confirmed the effectiveness of the dietary regimen for infesting the insect. Bacteria were isolated from all insects on LBA with concentrations that ranged from 10^5 to 10^9 CFUs per insect after the dietary treatment. Rifampicin-resistant bacteria were not recovered from untreated insects that were used as negative controls (i.e., not exposed to Sc 1-R). Microorganisms were not detected in the sterilized green beans or in the wash water used to rinse the surface-sterilized stink bugs. The highest concentration of Rif-resistant bacteria was 10^5 CFUs per insect from the treated stink bugs. Concentrations of Sc 1-R in male and female stink bugs were not significantly different ($P = 0.05$).

Insect Infestation on Cotton Bolls. In total, 192 caged southern green stink bug were used to fulfill a targeted 144 surviving insects (72 Sc 1-R contaminated and 72 control insects) after the 2-d caging period. Insect vigor was not apparently affected by Sc 1-R infestation. The highest bacterial concentration detected on the nonselective LBA medium was 10^9 CFUs per stink bug from untreated control males and 10^8 CFUs per insect from untreated control females. Bacteria were not recovered from control insects on LBA amended with Rif, whereas treated stink bugs exposed to Sc 1-R had a maximal bacterial concentration of 10^4 CFUs per insect.

Cotton Boll Evaluation. Bacteria were not detected from carpel imprints on LBA amended with Rif by using representative bolls tested before or after caging with any insect. From the bolls analyzed ($n = 188$) for signs of insect feeding, 151 had puncture wounds on the inner carpel wall and/or affected lint tissue. The number of punctures for all bolls caged with an insect ranged from 1 to 9 per boll with 97% of the bolls having 1 to 5 lesions. Both pierced and intact locule(s) were observed on individual bolls. Based on damaged lint and seed, 94% of the inner carpel lesions inflicted by control insects had no corresponding locule tissue damage. Conversely, all bolls that had been caged with treated insects and showed inner carpel damage exhibited at least seed discoloration (Figs. 1-3).

Bacteria were not detected from samples of control lint or seed tissue from insect-free bolls plated on nonselective media (Table 1). Overall, bacterial concentrations ranged from 10^2 to 10^9 CFUs/g tissue on LBA from all bolls with evidence of insect feeding/probing. Bacteria were recovered on LBA amended with Rif only from bolls caged with an insect that had been exposed to the Rif-resistant pathogen. Disease symptoms consistently corresponded with the detection of strain Sc 1-R based on selective medium growth.

Bolls Infested at 1 WPA. At 1 WPA, bolls exposed to a control insect had inconspicuous inner carpel blisters (Fig. 1), even though bacterial levels reached 10^7 CFUs/g locule tissue (Table 1) a week after the caging. Carpel blisters were pronounced on fruit harvested 2 and 3 WAE with up to 10^9 CFUs/g tissue; yet, locule damage ranged from minor discoloration to unaffected tissue due to feeding/probing (Fig. 1). At 1 WAE to treated insects, Sc 1-R levels attained 10^4 CFUs/g locule tissue, and disease symptoms were lim-

Fig. 2. Temporal effects of feeding/probing on greenhouse-grown cotton bolls by *N. viridula* infested with strain Sc 1-R or not harboring the bacterial cotton pathogen. Bolls were individually caged with a stink bug for 2 d at 2 wk post-anthesis. Samples were photographed at 1 (A and B), 2 (C and D), or 3 (E and F) wk after exposure to an insect. A, C, and E illustrate infection progression resulting from Sc 1-R transmission by stink bugs initially exposed to the opportunist via a contaminated food source. B, D, and F illustrate damage caused by stink bugs not harboring a cotton pathogen.

ited to the reddening of infected seeds. At this stage, seed coats were salmon-pink with embryos that were a darker red. A dark tan-brown discoloration spread over the entire infected locule by 2 WAE with a peak concentration of 10^7 CFUs/g locule tissue on the selective medium (Fig. 2). At 3 WAE, a 1,000-fold bacterial concentration decline was detected (Table 1). Locule(s) of infected bolls followed to yield were shrunken, completely blackened, and dense (Fig. 4A).

Bolls Infested at 2 WPA. Bolls caged with control insects at 2 WPA reached a maximal bacterial concentration of 10^4 CFUs/g locule tissue at 1 WAE (Table 1), with unpronounced blisters on the inner carpel (Fig. 2B). The highest bacterial levels from fruit harvested 2 and 3 WAE to control insects were 10^8 and 10^9 CFUs/g locule tissue, respectively (Fig. 2D and F), with clearly defined blisters but minimal effects observed on the lint and seed. At 1 WAE to treated insects, bolls infected with Sc 1-R had a high Rif-resistant bacterial concentration of 10^2 CFUs/g locule tissue and decayed seed with normal-seeming lint. Bolls infected for 2 wk contained deteriorated seed, discolored lint, and the highest level of Sc 1-R (10^8 CFUs/g locule tissue). Symptoms in bolls that had been infected for 3 wk were comparable with those described for samples at 2 WAE; however, the maximal Sc 1-R concentration decreased to 10^6 CFUs/g locule tissue. Infected locule(s) from bolls grown to yield were black, matted, and dense (Fig. 4B).

Bolls Infested at 3 wk WPA. Bolls at 3 WPA when exposed to control insects had an overall maximal bacterial level of 10^3 CFUs/g locule tissue at 1, 2, or 3 WAE (Table 1), with visibly unaffected lint and seed (Fig. 3). Locules from bolls infected with the stink bug vectored Sc 1-R for 1, 2, or 3 wk had typical lint coloration with seeds that were either apparently healthy, or discolored and arrested in embryo development (Fig. 3). The highest pathogen concentration detected 2 WAE (10^3 CFUs/g) was maintained at 3 WAE. Damage to locule tissue from cracked bolls infected with Sc 1-R was confined to the area immediately surrounding the puncture wound (Fig. 4C).

Discussion

This is the first study to temporally examine the effects to bolls resulting from southern green stink bug feeding and vectoring of a pathogenic *P. agglomerans*

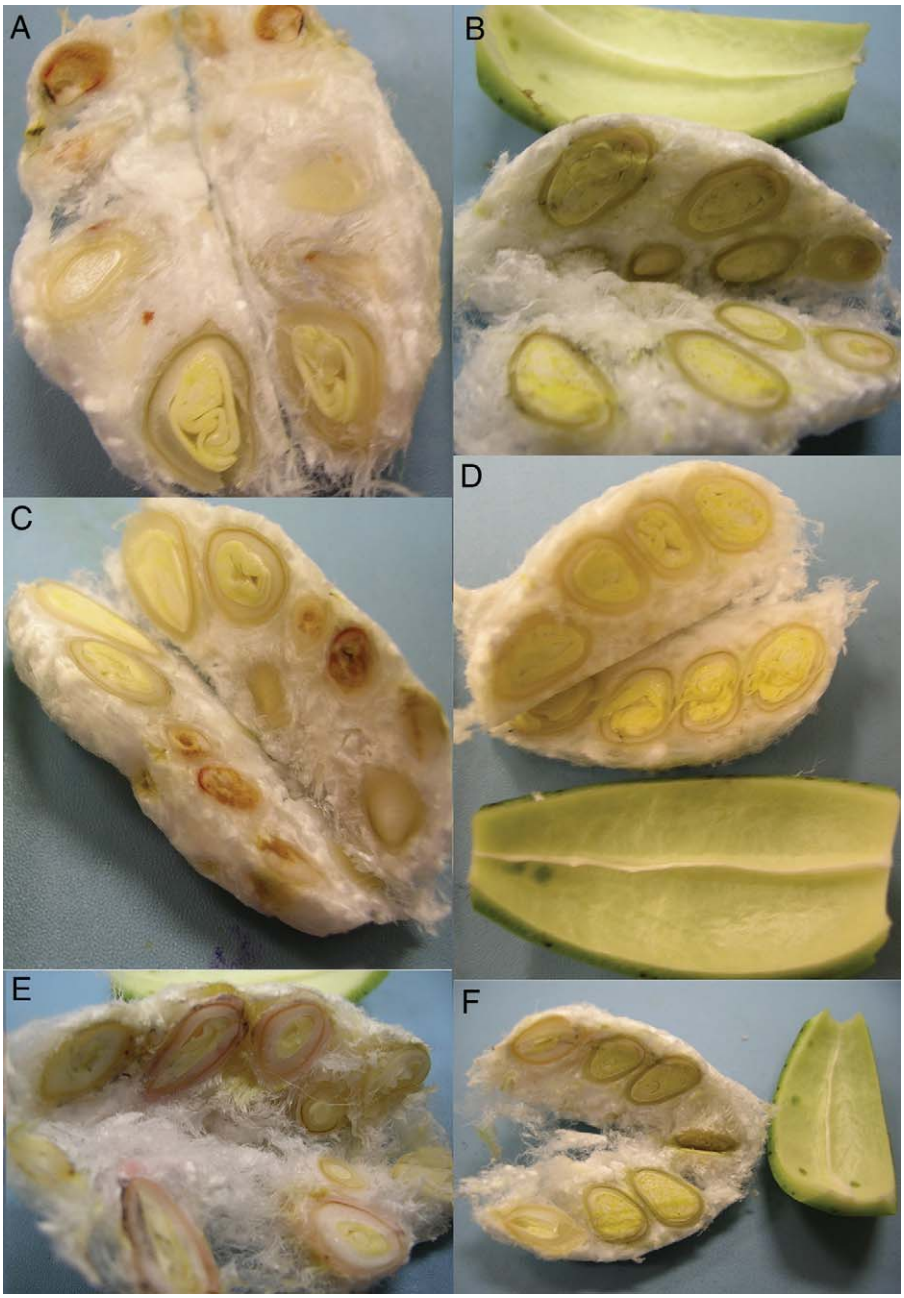

Fig. 3. Temporal effects of feeding/probing on greenhouse-grown cotton bolls by *N. viridula* infested with strain Sc 1-R or not harboring the bacterial cotton pathogen. Bolls were individually caged with a stink bug for 2 d at 3 wk post-anthesis. Samples were photographed at 1 (A and B), 2 (C and D), or 3 (E and F) wk after exposure to an insect. A, C, and E illustrate infection progression resulting from Sc 1-R transmission by stink bugs initially exposed to the opportunist via a contaminated food source. B, D, and F illustrate damage caused by stink bugs not harboring a cotton pathogen.

strain. Previously, causes of green boll damage and/or disease found in field samples have been generally associated with insect feeding injury, an unknown pathogen, or a hypothesized physiological disorder of certain full-season, transgenic cultivars. This study clearly defined stink bug injury inflicted by the piercing/sucking feeding mechanism as distinct from fruit infections caused by the vectored cotton opportunist *P. agglomerans*. We additionally illustrated that disease symptom expression produced by the pathogenic *P. agglomerans* strain depended both on the boll age when inoculated and the period of time after the infestation of the boll, thereby accounting for various symptoms observed among bolls at different nodes on field

Table 1. Range of concentrations of bacteria isolated from cotton locule tissue taken from control bolls and bolls fed upon at 1, 2, or 3 WPA by *N. viridula*

Harvest	Age of boll at initial exposure					
	One WPA		Two WPA		Three WPA	
	LBA[a]	LBA Rif[b]	LBA	LBA Rif	LBA	LBA Rif
	log CFUs/g tissue					
One WAE						
No insects (n = 9)	<1	<1	<1	<1	<1	<1
Insects, no Sc1-R (n = 24)	3–7	<1	2–4	<1	<1–2	<1
Insects, + Sc1-R (n = 24)	3–7	<1–4	2–5	<1–2	<1–3	<1–2
Two WAE						
No insects (n = 9)	<1	<1	<1	<1	<1	<1
Insects, no Sc1-R (n = 24)	4–9	<1	3–8	<1	<1–3	<1
Insects, + Sc1-R (n = 24)	4–9	<4–7	4–8	3–8	2–3	<1–3
Three WAE						
No insects (n = 9)	<1	<1	<1	<1	<1	<1
Insects, no Sc1-R (n = 24)	5–9	<1	5–9	<1	<1–2	<1
Insects, + Sc1-R (n = 24)	4–7	<2–4	2–7	<1–6	<1–2	<1–3

Insects were either fed or not fed a diet amended with *P. agglomerans* (strain Sc 1-R) and then individually caged with a boll for 2 d. The tissue was harvested 1, 2, or 3 WAE to the stink bugs.

samples from a given canopy. After a week of Sc1-R infection, irrespective of whether bolls samples were 1, 2, or 3 WPA at the point of inoculation, seeds were observed to be salmon-pink to red, and the lint seemed unaffected. The symptoms shown in this study are similar to those described in field study reports of the so-called South Carolina seed rot (Jones et al. 2000; Mauney and Stewart 2003, Mauney et al. 2004) and damage due to stink bug infestation (Greene et al. 1999; Willrich et al. 2004b). Thus, we propose that the current work provides a partial explanation to symptoms of boll damage described previously (Greene et al. 1999, Jones et al. 2000, Willrich et al. 2004b, Bommireddy et al. 2007) but does not preclude involvement of other pathogens in field-collected material.

In a prior publication, we reported detecting bacteria that included *Serratia*, *Staphylococcus*, *Pseudomonas*, *Pantoea*, and *Enterobacter* at levels reaching 10^9 CFUs/g tissue from locules with evidence of stink bug puncture (Medrano et al. 2007). This study corroborated our earlier observation because bacteria were readily deposited into bolls by southern green stink bugs during feeding/probing, suggesting that a fluid movement of microbes through southern green stink bug stylets is probable. Regardless of high concentrations of, in this case, unclassified bacteria, as compared with no bacteria detected ($<10^1$ CFUs/g tissue) in locules from noninfested control bolls, locules were asymptomatic. Furthermore, both nonpathogenic bacteria and Sc 1-R concentrations decreased as bolls developed. We hypothesize that the bacterial concentrations declined due to expenditure of the nutrients available in the developing locules. As early as 1 WPA, the fruit was tolerant to nonpathogenic bacterial contamination. Consistent with our previous report (Medrano et al. 2007), the effect of the nonpathogenic bacteria transmitted by stink bugs was localized in a small zone within only the penetrated locule. The lack of further bacterial distribution was also in accordance with our earlier study, and exceptions were only found in bolls that were 1 WPA when infested with the control insects.

Caging bolls at 2 WPA with control or treated insects resulted in effects after 2 WAE comparable with our previous observations (Medrano et al. 2007). At 1 WAE, a stink bug penetration of the inner carpel occurred as a water-soaked, point lesion, with other locule tissue apparently unaffected at the lint–endocarp interface. Even though nonpathogenic bacteria were transmitted by control insects and recovered from infiltrated locule tissue, dissected seed and lint

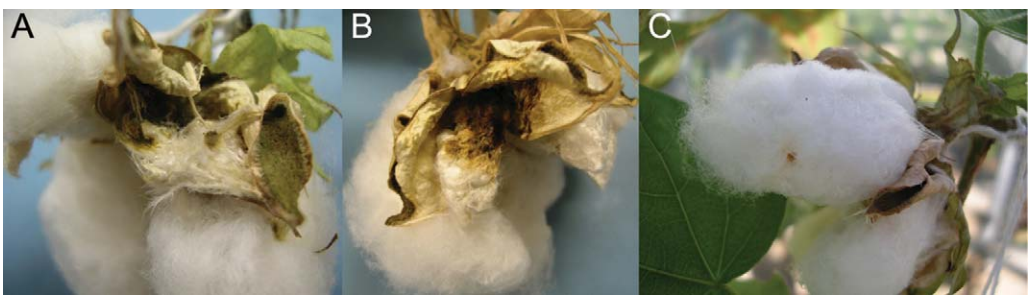

Fig. 4. Symptom expression of diseased cotton fruit resulting from the vectoring of an opportunistic *P. agglomerans* by *N. viridula* into bolls at different developmental stages. (A) Boll infected at 1 WPA. (B) Boll infected at 2 WPA. (C) Boll infected at 3 WPA.

tissue looked healthy. Water-soaked punctures by Sc 1-R–treated stink bugs after 1 WAE were undefined and just visible on the inner carpel, but they lacked damage on the surface of the locule at the point of endocarp contact. Appearance of infection symptoms ranged from reddening of the seed coat and/or embryo to necrosis of the entire locule and always corresponded with the detection of strain Sc 1-R.

Use of our disease model provided a method to systematically analyze boll damage resulting from southern green stink bug feeding/probing alone or in combination with infection by a vectored pathogen at various stages of fruit development. The model also provided a method to examine the progression of events after the exposure. Generally, bolls tolerated the trauma of stylet piercing through the carpel wall at 2 and 3 WPA with minimal effects evident at 1 WPA, suggesting that the detection of insects not vectoring a pathogen in the field would be of negligible concern to the grower. Similarly, the fact that bolls infected at 3 WPA restricted Sc 1-R to the point of stink bug inoculation indicated that pesticide applications may be unnecessary on fields where all harvestable bolls have reached this advanced stage (3 WPA), even if insects harboring an infective *P. agglomerans* are detected. Other studies focused on pest injury to cotton also have reported the resilience of bolls at 3 WPA and older (Willrich et al. 2004b; Bommireddy et al. 2007).

Results from these experiments indicate that damage associated with southern green stink bug infestations of green bolls is largely dependant on whether the insects transmit a cotton pathogen(s). Thus, these data premise the development of a tool that could be integrated into threshold management strategies to determine whether stink bugs harbor pathogens. Experiments to deduce a feasible system to determine whether pathogens are present in the insects are ongoing. In addition, research addressing the ability of stink bugs to harbor and transmit various opportunistic microbes is in progress.

Acknowledgments

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